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Modification of Rubisco and Altered Proteolytic Activity in O_3-Stressed Hybrid Poplar (*Populus maximowizii* × *trichocarpa*)[1]

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Exposing hybrid poplar (*Populus maximowizii* × *trichocarpa*) plants to ozone (O_3) resulted in an acceleration of the visual symptoms of senescence and a decrease in the activity and quantity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Whole plants, crude leaf extracts, and isolated intact chloroplasts of hybrid poplar clone 245 were used to test the hypothesis that O_3-induced structural modifications of Rubisco affect the activity of this key photosynthetic enzyme. Proteolytic activity, per se, could not account for losses in Rubisco; acidic and alkaline protease activities declined or were unaffected in foliage of O_3-treated poplar saplings. In vitro treatment of leaf extracts with O_3 decreased total Rubisco activity and binding of the enzyme's transition-state analog, 2-carboxyarabinitol bisphosphate. Additionally, O_3 increased the loss of Rubisco large subunit (LSU) when extracts were incubated at 37°C. Treatment of isolated intact chloroplasts with O_3 accelerated both the loss of the 55-kD Rubisco LSU and the accumulation of Rubisco LSU aggregates, as visualized by immunoblotting. The time-dependent modification in Rubisco structure was the primary response of the isolated organelles to O_3 treatment, with little proteolytic degradation of the LSU detected.

Exposure of plants to O_3 (ozone) often causes accelerated foliar senescence as characterized by premature leaf yellowing and abscission (Pell and Dann, 1991). In O_3-sensitive perennial species such as hybrid poplar (*Populus maximowizii* × *trichocarpa*), the accumulated effects of accelerated leaf casting over several years may lead to reduced productivity and decline of the organism (Pye, 1988). The phenomenon of O_3-induced accelerated senescence is accompanied by a premature decrease in activity and quantity of Rubisco. This effect has been observed for a variety of species, including wheat (Lehnherr et al., 1987), potato (Dann and Pell, 1989), radish (Pell et al., 1992), and hybrid poplar (Pell et al., 1992).

Recently, Mehta et al. (1992) demonstrated that oxidatively stressing wheat plantlets and intact chloroplasts from wheat with copper sulfate to induce senescence caused Rubisco protein to form cross-links with itself and be degraded at an enhanced rate. The authors suggested that through this mechanism the oxidative state of cells could contribute to the metabolic switch involved in initiating senescence. If such a relationship exists, environmental stresses that increase the oxidative load of organisms, such as exposure to O_3, could accelerate this process.

Although the specific factors that trigger senescence are not known, nutrient status may play an important role in foliar longevity (Kelley and Davies, 1988). As the enzyme responsible for photosynthetic carbon assimilation, Rubisco activity will affect the concentration of carbohydrates available for growth and repair. A decrease in Rubisco activity and/or quantity could possibly determine the point at which a leaf ceases to be an effective source of photoassimilate, setting into action the events leading to the breakdown and remobilization of the organ's components. To examine any possible role for Rubisco in mediating O_3-induced senescence, it is important first to know more about the mechanism by which Rubisco activity is altered by this oxidative stress.

O_3-induced decreases in Rubisco protein may involve an inhibition of protein synthesis and/or an increased rate of proteolysis. In mature leaves of hybrid poplar, the latter possibility may be of greater relevance because Rubisco synthesis decreases following leaf expansion (Dickmann and Gordon, 1975). Therefore, accelerated loss of Rubisco after leaf growth has been completed may primarily reflect O_3-stimulated proteolytic degradation. Increased proteolysis of Rubisco could be caused either by a quantitative increase in proteolytic enzyme activity or by modifications in Rubisco that make it a more acceptable substrate for proteases already present. Other environmental stresses, such as salinity in rice seedlings (Dubey and Rani, 1990) and water stress and SO_2 pollution in spruce (Pierre and Savouré, 1990), have been observed to alter protease activity. In these cases, stress increased the activity of some proteases and decreased the activity of others.

It has been proposed that protein modifications, such as oxidation by free radicals, identify proteins for hydrolysis (Dalling, 1987). In a number of experiments performed in vitro, oxidation has been shown to enhance the susceptibility of Rubisco to proteolytic degradation. Rubisco, purified from *Citrus*, became more susceptible to degradation by chymo-

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Abbreviations: CABP, 2-carboxyarabinitol-1,5-bisphosphate; LSU, large subunit of Rubisco; RuBP, ribulose-1,5-bisphosphate; SSU, small subunit of Rubisco.

trypsin after treatment with oxidative agents, with H_2O_2 being the most effective in promoting LSU degradation (Peñarrubia and Moreno, 1990). In *Lemna*, an oxidized form of Rubisco extracted from osmotically stressed fronds had increased susceptibility to exogenously added proteases in vitro (Ferreira and Davies, 1989; Ferreira and Shaw, 1989), although the rate of Rubisco turnover in the intact stressed fronds was not enhanced. Dann (1988) observed that treatment of purified potato Rubisco with O_3 increased the sensitivity of this enzyme to chymotrypsin action.

The objective of this study was to test two hypotheses for explaining O_3-induced loss of Rubisco activity: (a) O_3 increases the level of proteolytic activity in exposed foliage, resulting in the premature degradation of Rubisco and, consequently, reduced total activity; (b) O_3 or O_3-generated free radicals modify the Rubisco molecule such that it becomes inactivated. To test the first hypothesis, proteolytic activity was monitored throughout the life span of selected leaves on plants exposed to a chronic dose of O_3. Similar O_3 treatments have previously been shown to accelerate senescence and induce a decrease in Rubisco activity in hybrid poplar (Pell et al., 1992). Experiments addressing the second hypothesis evaluated changes in Rubisco protein patterns and total activity in crude leaf extracts and isolated intact chloroplasts exposed to O_3 in vitro. Experiments were performed with clone 245 of hybrid poplar. The foliage of this clone exhibited accelerated senescence in response to chronic O_3 exposures, while exhibiting minimal necrosis.

MATERIALS AND METHODS

Plant Culture

In 1990 and 1991, cuttings from hybrid poplar (*Populus maximowizii* × *trichocarpa*) clone 245 were collected and planted in 20-cm diameter containers (3.2 L of soil per container) as previously described (Pell et al., 1992). After bud break, plants were trimmed to one shoot, staked, and fertilized with 5.3 g of Sierra 17–6–10 (N-P-K) Plus Minors slow-release fertilizer (Sierra Chemicals, Milpitas, CA). During the spring and summer 1990 experiments, plants were additionally fertilized with 100 mL of a 3-g L^{-1} H_2O solution of Peter's Professional Water Soluble 20–20–20 (N-P-K) fertilizer (W.R. Grace Co., Cambridge, MA) before the start of the O_3 exposure. Plants in the spring 1990 experiment received a second treatment with soluble fertilizer, as above, 20 d into the exposure period. The saplings were cut back once to prevent them from growing into the tops of the exposure chambers (after 42, 18, and 40 d from the start of treatment in the spring 1990, summer 1990, and spring 1991 experiments, respectively). Plants received 85 μmol of photons m^{-2} s^{-1} of supplemental light from 600 to 2200 h throughout the experiments to ensure a 16-h photoperiod.

Whole-Plant O_3 Exposure

Approximately 1.5 months after bud break, plants were placed in continuously stirred tank reactors (Heck et al., 1978) for treatment with O_3 or charcoal-filtered air (controls). O_3 exposures were applied for 4 h d^{-1}, between 1000 and 1400 h, for every day of each experiment. In the spring 1990 experiment (initiated April 2, 1990), plants were exposed to 0.10 μL L^{-1} of O_3 on the first day of treatment, followed by 4 d at 0.08 μL L^{-1} and 55 d at 0.06 μL L^{-1} of O_3. In the summer 1990 (initiated June 12, 1990) and spring 1991 (initiated April 1, 1991) experiments, plants were exposed to 0.08 μL L^{-1} of O_3 for 29 and 60 d, respectively. O_3 was formed from O_2 as previously described (Enyedi et al., 1992).

The seventh or eighth leaf from the apex of each plant, corresponding to the youngest fully unrolled leaf, was initially tagged for subsequent harvests throughout the experiment. On each sampling date, one leaf from each of three plants per treatment was collected for dry weight measurement, extraction and analysis of Rubisco total activity, total soluble protein, proteolytic activity, and Rubisco protein quantitation (spring 1991 only). The specific activity of Rubisco (based on μmol of CO_2 fixed min^{-1} mg^{-1} of Rubisco protein) was determined on the second sampling date of each experiment.

Chloroplast Isolation and Characterization

Chloroplasts were isolated from the fully expanded leaves of hybrid poplar clone 245 after the plants had been placed under low light (maximum of 5 μmol of photons m^{-2} s^{-1}) for 2 d. The selection of leaves that were still supple was critical for obtaining sufficient yields of intact chloroplasts. Percoll gradients were generated 1 to 2 h before use by centrifuging 35 mL of a 50% (v/v) solution of 140 mL Percoll (Sigma), 4.2 g of PEG 8000, 0.14 g of BSA, 1.4 g of Ficoll in chloroplast medium (see below) at 40,000*g* in a Sorvall SS-34 rotor for 1 h and were refrigerated at 4°C until needed. Fresh leaf tissue, 30 to 50 g, was collected between 0900 and 1100 h and homogenized in a Waring blender with 300 mL of grinding buffer (0.35 M sorbitol, 50 mM Bicine/KOH [pH 8.0], 5 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1% [w/v] BSA, and 5 mM 2-mercaptoethanol) in 5-s bursts for a total of 20 s, filtered through four layers of cheesecloth followed by four layers of Miracloth, and centrifuged for 5 min at 2000*g* at 0 to 2°C. The pellets were resuspended in 12 to 16 mL of chloroplast medium (grinding buffer without BSA or 2-mercaptoethanol), applied to the top of Percoll gradients (3 to 4 mL per gradient), and centrifuged for 15 min at 9000*g* at 0 to 2°C. At the end of the centrifugation, the lower chloroplast band was collected, washed with 3 volumes of chloroplast medium, and resuspended in a final volume of 1 to 2.5 mL.

Chl was extracted from the chloroplasts in acetone (80%, v/v, final concentration) and measured by the method of Arnon (1949). The chloroplast-soluble protein to Chl ratio was typically between 8 and 10 (mg mg^{-1}). To establish an absence of vacuolar contamination, α-mannosidase activity was determined by the method of Boller and Kende (1979), except that 40 μmol of sodium acetate was substituted for succinic acid, and 0.2 μmol *p*-nitrophenyl α-D-mannopyranoside was used. Washed chloroplasts never contained more than 2.3% of the total α-mannosidase activity recovered from the gradient. In one test, latency of ferricyanide-dependent O_2 evolution (Lilley et al., 1975) indicated that chloroplasts prepared in this manner were nearly 100% intact. For most experiments chloroplast intactness was assessed using phase contrast microscopy. Preparations used for experiments con-

tained the same proportion of phase-bright chloroplasts as observed in the ferricyanide-tested sample (≥95%).

In Vitro O_3 Treatment

O_3 was generated from compressed air using an OREC O3VI ozonator. The O_3 stream was split between a Griffin O_3 monitor (model EG-2001-HC; Griffin Technics Corp., Lodi, NJ) and the delivery line. O_3 or compressed air was blown through an Eppendorf Combitip plastic syringe barrel over samples in 30-mL Corex tubes at a flow rate of 400 mL min^{-1}, with the tip of the gas-dispensing syringe positioned approximately 1 cm above the samples. This arrangement caused an indentation on the surface of the sample without creating bubbles or resulting in significant evaporation of the sample medium. All samples were maintained on ice throughout the gas treatment.

Crude leaf extracts for in vitro treatment with O_3 were prepared by homogenizing 0.5 g of fresh tissue from the youngest fully expanded leaves in 5 mL of extraction buffer (100 mM Bicine [pH 8.0], 0.2 mM Na_2EDTA, 20 mM $MgCl_2$, 20 mM $NaHCO_3$, 5 mM thiourea [an antioxidant; Van Driessche et al., 1984], and 1 mM 2-mercaptoethanol) and centrifuging for 10 min at 15,850*g*. The supernatant fluids constituted the crude leaf extracts used for experimentation. Four individual leaf extracts were divided into 1.2-mL volumes and treated with compressed air or O_3 in air (335–345 $\mu L\ L^{-1}$) at 0°C for 15 min. This dose was empirically found to be the minimum that produced the desired effects. After treatment, one part of each extract remained on ice, and the other was transferred to a 37°C bath for 30 min. At the end of the incubation period, all samples were returned to ice and analyzed for Rubisco total activity, Rubisco quantity, CABP binding, and total soluble protein.

Intact chloroplasts (equivalent to 50–200 μg of Chl) were treated with air or O_3 in air (335–345 $\mu L\ L^{-1}$) at 0°C as above for 1 h with gentle swirling on a gyratory shaker to keep the chloroplasts suspended. This treatment was found to be optimal for inducing the observed response without dehydrating the incubation medium. After treatment, samples were incubated on ice or in a 30°C bath for up to 48 h. Light levels never exceeded 4 μmol of photons $m^{-2}\ s^{-1}$ to prevent photooxidative damage. The incubation was terminated by adding 25 μL of SDS-PAGE sample buffer per 100 μL of chloroplasts and boiling for 1 min. This experiment was repeated three times.

Protein Quantitation and Immunoblotting

Protein extraction was accomplished by grinding foliar tissue in liquid nitrogen and homogenizing in extraction buffer (see above), 0.1 g of fresh weight mL^{-1}, using a Ten Broeck manual tissue grinder. Homogenates were centrifuged at 15,850*g* for 30 s, and the supernatant fluids were collected.

Soluble protein and total Rubisco protein were determined as previously described (Pell et al., 1992), except that a Pharmacia LKB Bromma-Ultroscan XL laser densitometer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) was used to scan proteins separated by SDS-PAGE. For electrophoresis, equal volumes of sample were applied to a 14% polyacrylamide separating gel (pH 8.8) with a 4.6% polyacrylamide stacking gel (pH 6.8).

For protein immunoblotting (Burnette, 1981), proteins separated as above were transferred to Zeta-Probe blotting medium (Bio-Rad) using a Bio-Rad Trans-blot electrophoretic transfer cell according to the manufacturer's directions, except that transfer buffer was diluted by 25% (v/v) with H_2O. Blots were probed with polyclonal antibody to hybrid poplar clone 245 Rubisco. Antibody was raised in New Zealand White rabbits according to the methods of Clausen (1974) except that RIBI trehalose dimycolate emulsion with monophosphoryl lipid A (RIBI Immunochem Research, Inc., Hamilton, MT) was used as the adjuvant system. Antibody against Rubisco LSU was affinity purified (Marchese-Ragona et al., 1988) from the antisera against Rubisco holoenzyme. Blots were developed using horseradish peroxidase linked to goat anti-rabbit immunoglobulin G, with 4-chloro-1-napthol as the colorogenic substrate (Harlow and Lane, 1988) in the presence of 0.015% (v/v) H_2O_2.

Activity Assays

Rubisco Total Activity

Rubisco total activity was determined by fixation of acid-stable ^{14}C using a procedure modified from Seemann et al. (1985). For the whole-plant experiments, 50 μL of leaf extract was combined with 400 μL of assay buffer (extraction buffer with 5 mM 2-mercaptoethanol) and the reaction initiated by adding 50 μL of RuBP (Sigma)/[^{14}C]Na_2CO_3 substrate (0.5 mM RuBP, 2.5 μCi of ^{14}C final concentration). The reaction was terminated after 30 s by the addition of 100 μL of 2 N HCl. Acid-stable ^{14}C was determined in a Beckman LS1701 scintillation counter. When Rubisco activity was determined for O_3-treated chloroplasts, assay buffer contained 10 mM DTT instead of 2-mercaptoethanol.

Proteolytic Activity

Proteolytic activity was assayed by the release of TCA-soluble ninhydrin-reactive material (Wittenbach, 1979) in the presence of 2 mg mL^{-1} of Rubisco (Sigma) substrate at pH 4.5 (in 80 mM citrate) or pH 7.8 (in 80 mM Bicine). Free amines were measured in 200 μL of a neutralized TCA-soluble fraction by adding 500 μL of ninhydrin reagent (Sigma), boiling for 20 min, and adding 5 mL of 25% (v/v) isopropanol. *A* was measured at 570 nm, and the NH_2 concentration was determined using a Leu standard curve (Moore and Stein, 1948).

CABP Binding

A modification of the procedure described by Collatz et al. (1979) was used to assess Rubisco substrate-binding capacity in the leaf extract studies. A 50-μL aliquot of leaf extract was combined with an equal volume of extraction buffer and 2 μL of [^{14}C]CABP (6 $\mu Ci\ \mu L^{-1}$) at 23 to 26°C. [^{14}C]CABP was made from [^{14}C]Na cyanide (Amersham) and RuBP (Sigma), as described by Collatz et al. (1979), and had a specific radioactivity of 0.02 mol Ci^{-1}. The mixture was briefly vortexed, and 500 μL of 2% (w/v) BSA in extraction buffer was

added; protein was precipitated with the addition of 300 μL of 60% (w/v) PEG 4000 and 100 μL of 0.2 M $MgCl_2$ (PEG 4000/$MgCl_2$) and vortex mixing for 5 s. After 30 min on ice, the samples were centrifuged at 15,850*g* for 3 min, the supernatant fluids decanted, and the pellets resuspended in 500 μL of extraction buffer. Protein was again precipitated with PEG 4000/$MgCl_2$ and centrifuged as before, and the protein pellets were resuspended in 500 μL of extraction buffer. Bound ^{14}C was counted, and CABP concentration was calculated from the specific radioactivity.

Data Analysis

Data from the whole-plant studies were analyzed by analysis of variance with O_3 treatment and day of exposure as class variables. All references to significant differences between treatments in "Results" were based on acceptance at the $P \leq 0.05$ level.

RESULTS

Whole-Plant Experiments

Very few of the leaves tagged for harvest exhibited symptoms of acute O_3 injury; leaves with lesions or stipple were excluded from the experiments. After 35 d of exposure in the spring 1990, 16 d in the summer 1990, and 15 d in the spring 1991 experiments, the oldest leaves on a number of the O_3-treated trees had started to senesce and eventually abscised, whereas no leaves were lost from control plants throughout the course of the experiments. If a leaf tagged for sampling abscised before harvest, it was included in the statistical analysis with a value of zero assigned for each parameter measured.

In both control and O_3-treated plants, Rubisco activity increased as the leaves expanded, peaked at approximately the point of reaching maximal leaf area, and then declined throughout the remainder of the experiment. Chronic exposure of hybrid poplar plants to O_3 caused a significant decrease in the total activity of Rubisco (Fig. 1A) in each of the three experiments performed. This effect was greatest during the early part of leaf life span, with Rubisco activities for the two treatments converging by day 40 of the exposure period in each of the two spring experiments and by day 23 in the summer 1990 experiment. Quantity of total soluble leaf extract protein (Fig. 1B) was not significantly affected by exposure to O_3 in any of the three experiments. The concentration of Rubisco protein was measured throughout the spring 1991 experiment only (Fig. 1C). Although Rubisco protein content was apparently decreased by O_3 on day 20, coinciding with the time when O_3 had the greatest effect on Rubisco activity, this effect was not statistically significant at the $P \leq 0.05$ level. In all three experiments, O_3 reduced Rubisco specific activity (decreases ranged between 9 and 30% of the specific activity in control plants) when it was measured on the second sampling date of each experiment (data not shown).

Immunoblot analysis was performed on foliar proteins separated by SDS-PAGE from day-20 extracts of the spring 1991 experiment (Fig. 2B). The only protein band reacting with antibody to Rubisco LSU corresponded to the 55-kD Rubisco LSU.

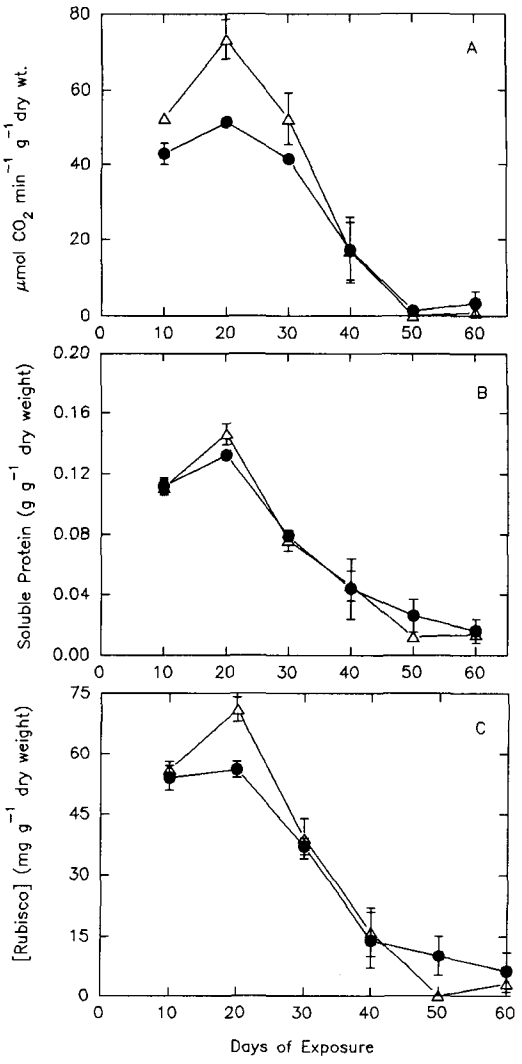

Figure 1. Effect of chronic O_3 exposure on Rubisco activity (A), concentration of soluble protein (B), and quantity of Rubisco protein (C) in hybrid poplar foliage. This experiment was replicated three times; one representative graph (spring 1991) for each parameter is shown. Each point represents the mean of samples from three randomly selected plants, and vertical bars represent SE. Δ, Control; ●, O_3 treated.

Proteolytic activity at pH 4.5 was significantly reduced in O_3-treated plants during the spring 1990 and spring 1991 experiments (Fig. 3A). Proteolytic activities of leaf extracts at pH 7.8, measured only for the summer 1990 and spring 1991 experiments, were not significantly changed by exposure to O_3 (Fig. 3B).

In Vitro Experiments

Leaf Extracts

Crude leaf extracts were treated with either compressed air or O_3 for 15 min at 0°C, followed by incubation for 30 min

at 0 or 37°C. At 0°C, O_3 treatment had little effect on LSU concentration but decreased Rubisco activity by 48% and CABP binding by 23% (Table I). Air treatment followed by incubation at 0°C had no effect on LSU concentration, Rubisco activity, or CABP binding. At 37°C, LSU concentration decreased by 29% in O_3-treated extracts compared to about a 5% decrease in air-treated extracts.

Immunoblot analysis of soluble proteins from treated leaf extracts demonstrated that in addition to the 55-kD Rubisco LSU protein band, two high molecular mass protein bands reacting with affinity-purified antibody to Rubisco LSU appeared in O_3-treated samples (Fig. 4B). Incubation at 37°C enhanced the intensity of these high molecular mass bands. None of the other protein bands, including Rubisco SSU, cross-reacted with the antibody.

Chloroplasts

When isolated intact chloroplasts were treated with O_3 for 1 h at 0°C, Rubisco activity decreased by a mean value of 27% from that measured in nontreated chloroplasts maintained on ice for the same length of time (data not shown). Rubisco activity in air-treated chloroplasts decreased by just 4% (data not shown). O_3 had very little effect on Rubisco LSU concentration when chloroplasts were not incubated after treatment (Fig. 5A, 0 h). However, when the chloroplasts were incubated for up to 48 h following gas exposure, qualitative differences in LSU were observed between treatments. With incubation at 30°C, pretreatment with O_3 resulted in an accelerated loss of the 55-kD LSU. As the 55-kD LSU declined, there was a concomitant increase in a protein band with a molecular mass larger than 205 kD on SDS-PAGE gels stained with Coomassie blue (Fig. 5A). Immunoblot analysis demonstrated that the >205-kD band, actually a doublet, reacted with antibody to Rubisco LSU (Fig. 5B). Two other bands, corresponding to proteins of approximately 65 and 110 kD, also reacted with antibody to LSU. At 0°C, these high molecular mass bands were detected only in O_3-treated chloroplasts. At 30°C, these aggregated forms of Rubisco were present in air-treated chloroplasts as well but to a lesser extent than observed for the O_3-treated chloroplasts. Cross-reaction of the Rubisco antibody with the molecular weight marker shown in this blot is attributed to a possible contamination of the marker with Rubisco or sample protein.

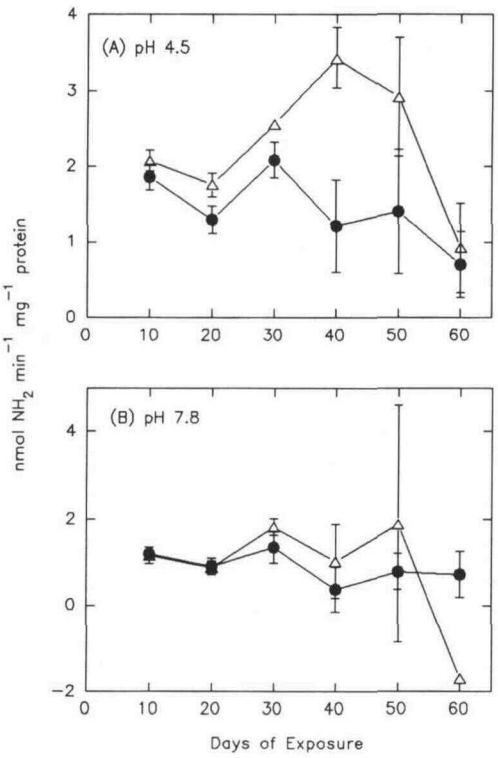

Figure 3. Proteolytic activity from control and O_3-treated hybrid poplar foliage extracts incubated at pH 4.5 (A) and pH 7.8 (B). The pH 4.5 experiment was replicated three times, and the pH 7.8 experiment was replicated twice; one representative graph (spring 1991) is shown for each experiment. Each point represents the mean of samples from three randomly selected plants, and vertical bars represent the SE. △, Control; ●, O_3 treated.

DISCUSSION

Previous research conducted with hybrid poplar clone 388 demonstrated that O_3-induced decreases in net photosyn-

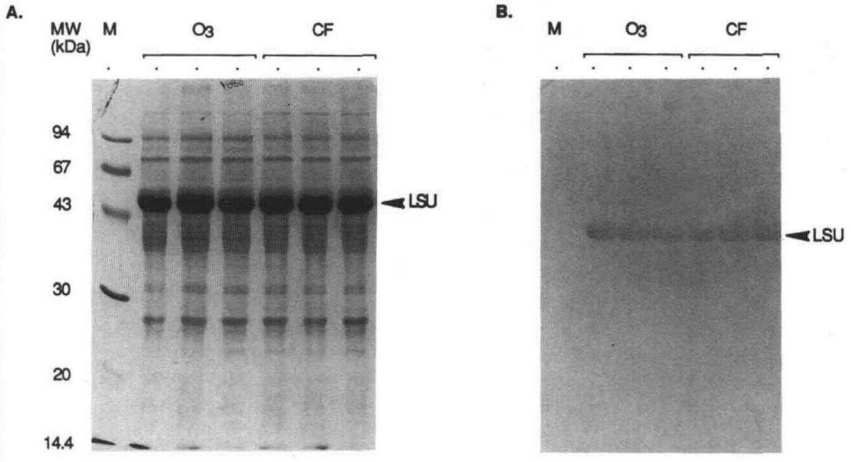

Figure 2. Soluble protein and Rubisco banding patterns in leaf extracts of hybrid poplar clone 245 treated with O_3 for 20 d. Leaf extracts from the spring 1991 whole-plant experiment (see Fig. 1) were analyzed by SDS-PAGE and protein immunoblotting. A, SDS-PAGE gel stained with Coomassie blue. CF, Control plant extracts; O_3, O_3-treated plant extracts. Equal volumes of extract were applied to each of the respective three lanes, corresponding to an average protein concentration of 12.5 μg. Molecular mass markers (M) are shown in the left-most lane. Rubisco LSU is indicated on the right. B, Immunoblot of companion gel to A using affinity-purified antibody to poplar Rubisco LSU.

Table I. *Total soluble protein (Protein), LSU concentration (LSU), CABP binding (CABP), and total activity of Rubisco (Activity) in hybrid poplar leaf extracts treated with compressed air or O_3*

Gas treatment was conducted for 15 min at 0°C. Incubation was conducted for 30 min following gas treatment at 0 or 37°C.

Measured Parameter	Air		O_3	
	0°C	37°C	0°C	37°C
Protein	99.9 (1.9)[a]	98.6 (4.7)	96.7 (4.6)	96.0 (4.2)
LSU	101.3 (6.5)	95.5 (2.0)	94.3 (3.6)	71.4 (10.7)
CABP	97.6 (15.2)	79.6 (8.4)	77.3 (5.8)	41.5 (9.7)
Activity	103.6 (5.0)	45.3 (18.8)	52.4 (14.8)	18.1 (7.8)

[a] Data are expressed as percentages of the measurements for nongassed extracts incubated at 0°C for 45 min. Values are the means of four replicate experiments; numbers in parentheses represent SE.

thesis were paralleled by decreases in Rubisco activity (Pell et al., 1992), suggesting that this enzyme may become limiting to photosynthesis during episodes of O_3 exposure. In the experiments described here, hybrid poplar clone 245 exhibited significant reductions in total Rubisco activity in response to O_3 stress. The largest effect of O_3 on total Rubisco activity in clone 245 took place early in the experiment (Fig. 1A), at approximately the point in foliar development when full expansion was achieved (data not shown). This research has focused on two possible causes for the observed decrease in Rubisco activity: (a) decreased concentration of Rubisco protein due to proteolytic degradation and (b) structural modification of the Rubisco molecule resulting in enzyme inactivation.

Previous studies have shown that at a higher daily dose of O_3 (0.10 μL L^{-1} for 4 h d^{-1}), Rubisco quantity in hybrid poplar clone 388 closely reflected Rubisco total activity (Pell et al., 1992). At the dose of O_3 used in the whole-plant experiments described here (0.06 or 0.08 μL L^{-1} for 4 h d^{-1}), Rubisco protein concentration was not significantly affected (Fig. 1C). At just one time early in the spring 1991 experiment, corresponding to the time of maximal O_3 effect on Rubisco activity, was Rubisco protein concentration lower in O_3-treated plants than in controls. Rubisco specific activity at this time was decreased in leaf extracts of the O_3-treated plants (data not shown), indicating that Rubisco activity declined to a greater extent than enzyme concentration.

Proteolytic activity was analyzed in these experiments to determine whether or not O_3 may cause an increase in protease activity, perhaps accounting for the reduction in Rubisco activity via proteolytic damage to the enzyme. To the contrary, we found that proteolytic activity was either reduced or unaffected in the foliar extracts of treated plants, depending on assay pH (Fig. 3). It is possible that O_3 may have induced subtle increases in protease activity against Rubisco that were not detectable by the methods used. The lack of low molecular mass protein bands corresponding to Rubisco LSU degradation products on the immunoblots (Fig.

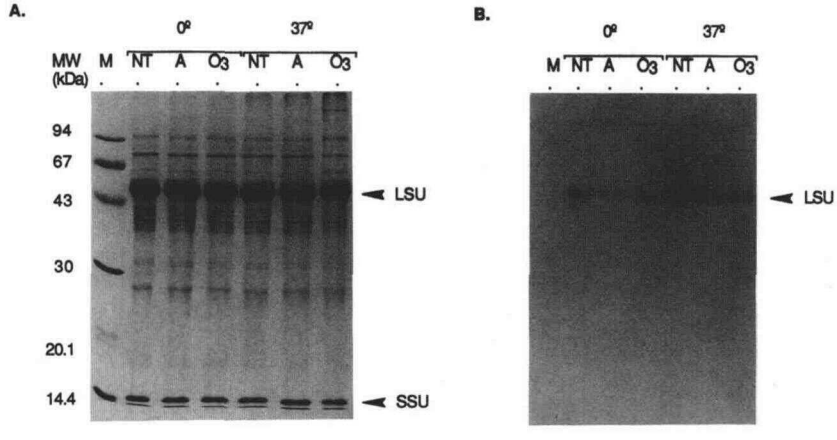

Figure 4. Effect of O_3 on crude leaf extract soluble protein. Equal volumes of leaf extract sample, corresponding to an average concentration of 5.1 μg of protein/lane (range, 4.9 to 5.3 μg protein/lane), were analyzed by SDS-PAGE and immunoblotting. A, Coomassie blue-stained gel. NT, Extracts without gas treatment incubated at 0°C for 45 min; A, extracts air treated for 15 min at 0°C, followed by incubation at 0 or 37°C for 30 min; O_3, extracts O_3 treated for 15 min at 0°C for 15 min, followed by incubation at 0 or 37°C for 30 min. Molecular mass markers (M) are shown in the leftmost lane. Rubisco LSU and SSU are labeled on the right. B, Immunoblot of companion gel to A using affinity-purified antibody to poplar Rubisco LSU.

2B) could indicate either that no breakdown was taking place or that proteolysis of Rubisco resulted in rapid degradation to immunologically nonreactive fragments.

Taken together, these results do not support the hypothesis that O_3 decreases Rubisco activity via the induction of proteases and extensive Rubisco degradation. The reductions in Rubisco specific activity suggest, instead, that, under the conditions of these experiments, Rubisco is modified in a manner that decreases its function before loss of enzyme concentration.

The in vitro experiments with crude leaf extracts demonstrated that, either directly or via free radicals created from reactions with chemical reagents and/or biological molecules (Grimes et al., 1983), O_3 damaged Rubisco under conditions in which treatment with air had virtually no effect (Table I and Fig. 4). CO_2-fixing activity was most sensitive to O_3 treatment; capacity for binding CABP, a six-carbon transition-state analog for Rubisco (Pierce et al., 1980), was reduced to a lesser extent (Table I). Incubation at 37°C promoted the loss of the 55-kD LSU in the O_3-treated extracts, but no low molecular mass degradation products reactive with antibody to LSU could be detected by immunoblot analysis (Fig. 4B). Autoproteolytic activity against Rubisco in tissue extracts is dependent on pH and the developmental age of the plant tissue (Cercós et al., 1992). It is possible that optimal conditions for autodigestion were not met in this experiment with extracts from young leaves incubated at pH 8.0.

Loss of the 55-kD LSU may be partially explained by the appearance of high molecular mass proteins reactive with antibody to LSU. These proteins probably represent Rubisco that has aggregated or cross-linked with itself or other proteins due to oxidative modification. Slight modification of Rubisco caused by O_3 may result in lost enzyme function, whereas the most damaged proteins are denatured and/or degraded.

The in vitro experiments with isolated chloroplasts allowed us to treat poplar Rubisco with O_3 in the absence of cytoplasmic and vacuolar proteases and had the added benefit of maintaining Rubisco in its in situ environment during O_3 exposure. The results of these experiments indicated that, although O_3 decreased Rubisco activity by about 27%, there was little capacity for degradation of the protein by the isolated organelles. As in the experiments with leaf extracts, O_3 treatment accelerated the appearance of high molecular mass aggregates of the Rubisco LSU on SDS-PAGE gels (Fig. 5B). If these aggregates are composed entirely of Rubisco protein, they could account for all of the decrease in the 55-kD LSU (data not shown). Extrachloroplastic or age-dependent factors required for proteolysis may have been lacking or present in suboptimal quantities in the isolated organelles, or aggregation may cause the protein to be resistant to proteolytic degradation. It is also possible that O_3 treatment may have inactivated chloroplastic proteases involved in degrading the aggregate.

The appearance of high molecular mass aggregates of Rubisco LSU in the in vitro systems indicates that O_3 has the capacity to make changes in Rubisco protein structure, making it more susceptible to cross-linkage with itself and/or other proteins. The absence of Rubisco LSU aggregates in leaf extracts from hybrid poplar treated with chronic exposure to O_3 (Fig. 2B) could mean that this phenomenon is an artifact of the conditions of the in vitro experiments, i.e. the presence of chemical reagents in crude leaf extracts, large doses of O_3 delivered in a short period of time. These important differences prevent a direct comparison of the in vitro and in vivo experiments. A demonstration that O_3 induces the formation of Rubisco aggregates in vivo would be needed to establish the physiological significance of such modifications.

In these studies we have reaffirmed that when hybrid poplar plants are exposed to O_3 they exhibit an accelerated

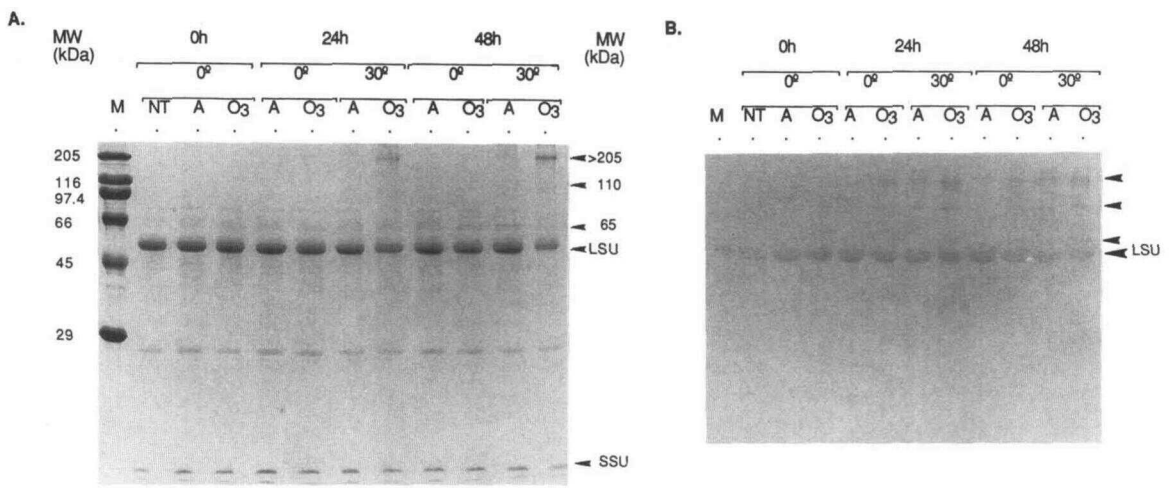

Figure 5. O_3-induced changes in isolated chloroplast protein and Rubisco. A, Coomassie blue-stained chloroplast proteins analyzed by SDS-PAGE. NT, Chloroplasts without gas treatment incubated at 0°C for 1 h; A, chloroplasts treated with compressed air for 1 h at 0°C before incubation; O_3, chloroplasts treated with O_3 for 1 h at 0°C before incubation. Incubation was on ice (0°C) or at 30°C for 0, 24, or 48 h. Molecular mass markers (M) are shown in the left-most lane. Positions of Rubisco LSU and SSU are shown by arrowheads on the right. B, Immunoblot with antibody to poplar Rubisco LSU performed on a companion gel identical with the one shown in A. Rubisco LSU is labeled on the right, and aggregates (>205, 110, and 65 kD) are indicated with arrowheads on the right.

rate of senescence. O_3 treatment decreased Rubisco activity but did not significantly lower Rubisco protein concentration or enhance proteolytic activity at the two pH values tested. O_3 treatment conditions that inactivated Rubisco in vitro caused a cross-linking modification of the enzyme without measurably increasing the susceptibility of Rubisco to degradation. The relationship between these effects and the loss of the protein in vivo are under study in our laboratory.

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